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Administered Sustained Release Vaccines

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13. ABSTRACT (Maximum 200 words) This research focuses on the development of an orally administered microencapsulated vaccine against botulinum neurotoxin type A using the non-toxic C-fragment of the neurotoxin (Bot-Ac). The research focuses on the production through recombinant techniques and the incorporation of a known bioadhesive, vitelline protein B (vpb), into the microsphere preparations to enhance uptake of the microspheres by the M cells located in the Peyer's patches. The results demonstrate that vpb can be produced at 175-350 mg/L in a Pichia pastoris expression system. Also, microspheres that contain 10% vpb generate a higher serum IgG level than microspheres that do not contain vpb when administered either orally or subcutaneously. Whether vpb is acting as a true bioadhesive is still under study through the use of Peyer's patch uptake experiments. For comparison it was also determined that when 2.0 mg of 10 mm microspheres containing 89.9% BSA / 10.0% vpb / 0.1% Bot-Ac were administered subcutaneously, a detectable serum IgG level was generated at 17 weeks post-immunization.				
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FOREWORD

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1. Introduction

Vaccines have tremendous dual use potential for both military and large scale civilian immunization programs such as the Children's Vaccine Initiative (CVI) sponsored by the World Health Organization, Unicef, United Nations Development Fund, Rockefeller Foundation, and World Bank¹. The ideal vaccine would: i) be stable enough to be stored at room temperature, ii) provide lifelong protection with a single dose, iii) and be orally administered.

Microencapsulation of vaccine antigen is a promising method to achieve all three of the above criteria. Since most vaccine antigens are proteinaceous, microencapsulation would stabilize the antigen against a variety of degradative reactions that typically plague protein preparations including deamidation of asparagines and glutamines, oxidation of tryptophans, methionines and disulfides, hydrolysis of the peptide backbone, and many others. Therefore, antigen protection by microencapsulation would increase shelf life and possibly eliminate the cold chain requirement of most vaccine preparation². Microencapsulation of antigen can also eliminate the need for booster vaccine administration since microcapsules can be prepared to provide a timed-release of antigen over a period of months. The exposure of the immune system to a constant low dose of antigen will frequently generate a potent and long lasting immune response. Finally, if the microspheres were bioadherent and of the appropriate size ($< 10 \mu\text{m}$) they would "stick" to the intestinal lining and be phagocytized by M cells in the Peyer's patches and ultimately induce both a mucosal and systemic immune response^{3,4}. This dual immune response would provide a primary and secondary immune defense against pathogens and toxins that invade the body via mucosal surfaces^{2,3}. In addition, orally administered vaccines can overcome the problems associated with parenteral vaccines, such as infection and the required administration by trained medical personnel⁵. This research focuses on the development of a biodegradable, bioadherent, orally administered vaccine against botulinum neurotoxin type A using the non-toxic C-fragment of the neurotoxin (Bot-Ac).

In this research Bot-Ac is encapsulated in biodegradable proteinaceous microspheres. The microspheres consist of bovine serum albumin (BSA) and recombinant vitelline protein B (vpB) from the liver fluke *Fasciola hepatica* which is a known bioadhesive^{6,7,8}. The encapsulation method is the classical, well described water in oil technique for the preparation of protein-based microspheres⁹. The encapsulated Bot-Ac is either subcutaneously or orally administered to Balb/c mice and antibody titers in both sera and saliva are measured.

Microspheres composed of other bioadhesive materials are also being considered. Chitosan [(1 \rightarrow 4) 2-amino 2-deoxy- β -D-glucan] is a polyaminosaccharide, normally obtained by alkaline deacetylation of chitin which is a very abundant naturally occurring polymeric material. It occurs as a principle constituent of the protective cuticles of crustacea and insects and also in the cell walls of some fungi and microorganisms. Chitosan is a very versatile polysaccharide and has been the focus of much research. Of particular interest to this project is chitosan's bioadhesive characteristic¹⁰ and its application toward a bioadhesive oral formulation.

2. Materials and Methods

2.1. Materials

vpB is expressed in *Pichia* yeast and isolated and purified as described below. Bot-Ac was purchased from Ophidian Pharmaceuticals. The primary monoclonal was purchased from Sigma, and secondary polyclonals conjugated to horseradish peroxidase, from Biogenesis, Inc. Corning 96 well ELISA microtiter plates were purchased from Fisher. 2,2'-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) was purchased from Pierce. BSA, 25% glutaraldehyde in 1 ml ampoules, olive oil, castor oil, hexadecane, Tween-20, buffers and other miscellaneous items were purchased from Sigma.

2.2. BSA Microsphere Preparation

All BSA-based microspheres were prepared by the water in oil emulsion technique using glutaraldehyde crosslinking as previously described¹¹. Briefly, 125 ml of oil is stirred at 1,200 rpm for 30 minutes. Then 2 ml of a 150 mg/ml protein solution containing BSA, vpB, and Bot-Ac is added to the oil and stirring is continued for 15 minutes. The aqueous droplets in the emulsion are solidified by adding 0.5 ml of an aqueous glutaraldehyde solution (1 to 25% glutaraldehyde). The emulsion is stirred for an additional 15 minutes. Then 50 ml of acetone is added and the emulsion is stirred for an additional 10 minutes. The microspheres are then collected by centrifugation at 3,000 rpm for 10 minutes. The supernatant is decanted and discarded and the pelleted microspheres are resuspended in 50 ml acetone to remove residual oil. The centrifugation and acetone washes are repeated twice more. The microspheres are allowed to air dry overnight at room temperature to allow the remaining acetone to evaporate. Yields of the free flowing microspheres ranged from 240 to 288 mg of microspheres from 300 mg of starting protein (i.e 80 to 96%).

Attempts to prepare 100% vpB microspheres failed. As described in the Results section, large amounts of vpB (> 200 mg) are difficult to obtain and the maximum solubility of the protein at neutral pH appears to be relatively low (~20mg/ml). Both of these reasons have hindered the preparation of 100 % vpB microspheres.

2.3. Preparation of BSA Nanospheres

The preparation of BSA nanospheres is essentially the same as previously described¹². Briefly, 30 ml of 10 mg/ml BSA, pH 5.55 at room temperature was added to 50 ml of 90°C deionized water with slow stirring via a magnetic stir bar. The emulsion formed immediately and was then frozen and lyophilized to dryness overnight.

2.4. Determination of Microsphere Size and Surface Morphology

The size and surface morphology of the microspheres were determined by a Jeol T330A scanning electron microscope at the Electron Microscopy Center on the campus of Texas A&M University. Microspheres were coated with 20 nm of gold-palladium using a sputter coater in an

argon atmosphere with a digital thickness monitor. The approximate average diameter of dry microspheres was determined qualitatively for each batch by visual inspection of the micrographs. Surface morphology was also determined qualitatively.

2.5. *In Vitro* Release Kinetics

The *in vitro* release assay was performed in duplicate for each sample as follows: 7 mg of microspheres were suspended in 70 μ l of acetone and 630 μ l of 10 mM PBS, pH 7.4. At various time points the microspheres were again pelleted to collect the supernatant and it was replaced with an equal volume of fresh PBS. The protein concentration of each supernatant aliquot was determined by the BCA protein assay (Pierce) and the total amount of protein released from the microspheres to that time was determined.

2.6. Microsphere Administration to Balb/c Mice

Ten-week-old Balb/c female mice weighing ~ 18 g were administered microsphere formulations either orally or subcutaneously. Oral doses were administered in 200 μ l of 98% 10 mM PBS, pH 7.4 / 2% ethanol using a curved tube style 1.5 inch, 20 gauge feeding needle attached to a 1 ml Becton Dickinson syringe with Leur-Lok tip and 0.05 ml graduations. Subcutaneous doses were administered between the shoulder blades and distal to the head in the same buffer using a 3/8", 26 gauge needle attached to the same syringe described above.

2.7. Determination of Antibody Response to Bot-Ac

To determine serum antibody response to Bot-Ac, blood was collected from the tail vein of all mice at the desired time point into heparinized microcapillaries. Blood from all mice in the same group was pooled and centrifuged to collect the sera. The sera was frozen at -20°C until the ELISA was performed. To determine saliva antibody response to Bot-Ac, salivation was induced by injecting 100 μ l of 1 mg/ml pilocarpine interperitoneally. After a few minutes, 100 μ l of saliva was collected from the mouth of each mouse in a microcapillary. Saliva from all mice in the same group was pooled, centrifuged to clarify and frozen at -20°C until the ELISA was performed. Saliva collection, when performed, was done after blood collection. The presence of antibodies against Bot-Ac was determine using the following ELISA:

96 well polystyrene microtiter plates were coated with 100 μ l/well of 10 μ g/ml Bot-Ac diluted in 0.05 M carbonate buffer pH 9.6 (1 μ g Bot-Ac/well). The plates were incubated overnight in a humid chamber with shaking at room temperature to allow Bot-Ac to bind to the plates. The Bot-Ac solution was discarded and the plates were washed four times with PBS containing 0.05% Tween-20 pH 7.4. The plates were inverted and tapped to remove excess PBS between each wash. The serum samples collected from the mice were diluted 1:4 with PBS containing 0.05% Tween-20. The plates were incubated with the diluted sera for two hours at 37°C. The diluted sera was discarded and the plates were washed twice with PBS containing 0.05% Tween-20. Then goat anti-mouse IgG or anti-mouse IgA conjugated to horseradish peroxidase diluted 1:1000 with PBS/Tween was added to the wells and incubated for one hour at 37°C. The secondary antibody solution was discarded and the plates were washed five times

with PBS containing 0.05% Tween-20. The plates were inverted and tapped to remove excess buffer between each wash.. Then 100 μ l of ABTS was added in 0.05 M phosphate/citrate buffer (pH 5.0) containing 0.03% sodium perborate. The resulting color development was determined after 30 minutes at 405 nm. Data was then corrected by subtracting the negative control absorbance (normal mouse sera).

3. Results

3.1. Production of vpB Protein through Batch Fermentation

During the last funding period, work has continued toward improving the protein yield of vpB produced by *Pichia* transformants by monitoring and modifying various parameters of the fermentation process which affect yeast cell growth and consequently impact protein production. Efforts have focused in three areas: optimization of biomass prior to induction of vpB protein, optimization of methanol induction, and stabilization of the secreted protein during the fermentation process.

Pichia pastoris, a methyltrophic yeast strain, was selected for transformation with recombinant pPIC9 vector carrying the *F. hepatica* gene encoding vpB. In addition, pPIC9 carries the secretion signal from the *S. cerevisiae* alpha-mating factor pre-pro peptide such that, in the presence of the inducer methanol, heterologous protein secretion into the culture supernatant occurs (see Figure 1 for graphic of pPIC9). *Pichia* itself secretes very low levels of native proteins and, once transformed, tolerates growth in minimal growth media.

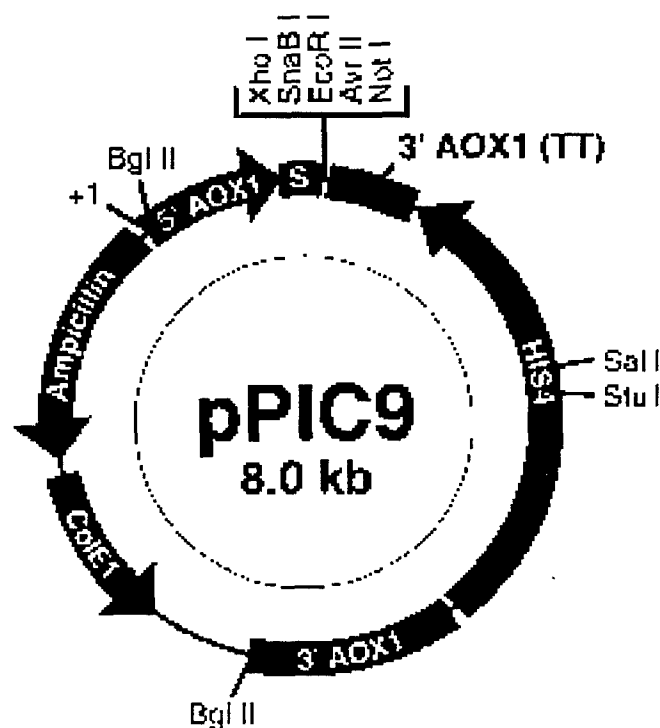


Fig. 1. Map of pPIC9, reprinted from Invitrogen instruction manual.

Therefore, the vast majority of the total protein recovered from the culture media should be the expressed foreign protein (or vpB). There are several examples of proteins which were successfully expressed in *Pichia pastoris* that were completely unsuccessful in baculovirus or *S. cerevisiae* suggesting that the *P. pastoris* system represents a superior protein expression and production system.

The process of heterologous protein expression in *Pichia* is carried out using a 1.5 L mini-fermentor. To generate the fermentor inoculum, 10ml cultures of *Pichia* (transformed with pPIC9-vpB) were grown overnight (at 27°C) in 250 ml baffled flasks containing MGY medium to an OD₆₀₀ = 10-20. At this point, the 10 ml cultures were transferred to 2 L flasks containing 500ml fresh MGY media. Incubation was continued for 24 hours at 27°C, or until the OD₆₀₀ reaches 2-6. Once the cells reach mid-log phase growth, the culture was interrupted by centrifugation at 5000 rpm for 15 minutes to pellet the cells. The cell pellet was resuspended in a final volume of 1.5 L fresh MGY media and the entire culture was transferred to the mini-fermentor. Culture conditions in the fermentor included vigorous agitation (300 rpm), aeration (50-75 L/hr air) and 27°C. Growth under these conditions was continued for approximately 72 hours in the fermentor; once daily, the culture was supplemented with the addition of a 10% glycerol solution (final concentration=0.5%) in an effort to boost cell density prior to inducing foreign protein expression. This "mixed feed" approach (continued growth in the presence of glycerol prior to and along with the subsequent addition of the inducer methanol) has been observed to enhance the expression of certain foreign proteins by *Pichia*. Once the fermentor culture OD₆₀₀ reached a level of 40-60, the process of induction was initiated with the daily introduction of 100% methanol (final concentration = 0.5%) to the fermentor. vpB production generally occurs by 120 hours post-induction. In an effort to eliminate this delay in the induction process, the "mixed feed" strategy was employed and resulted in vpB expression as early as 24 hours post-methanol introduction. Clearly, the higher the biomass achieved prior to the addition of methanol, the greater the likelihood for enhanced protein production. Throughout fermentation, 1 ml samples were removed daily to monitor yeast cell growth and viability, culture pH, and foreign protein production and secretion.

After biomass scale-up through fermentation and the mixed feed strategy, a drop in the vpB protein yield was still noted. One possible explanation was that the vpB protein, although expressed and secreted into the culture media, was subject to proteolysis. As a result, unbuffered media (MMY) is currently used which allows the yeast culture byproducts to gradually acidify the media to a pH of approximately 3-4. Under low pH conditions, proteases present in the media are inactivated and vpB is stabilized in its intact form.

Following sufficient methanol induction of the *Pichia* fermentor culture, vpB protein is purified and concentrated by ammonium sulfate precipitation. Exhaustive dialysis in several changes of 1X PBS rids the protein solution of excess salt. The ammonium sulfate purified and dialyzed fraction containing the greatest amount of vpB protein (generally, corresponding to 40-60% ammonium sulfate) was concentrated using the Amicon Centriprep Concentrator (MW cut-off 10,000).

Approximately 250-500 mg of vpB protein is routinely generated from a 1.5 L fermentation experiment. When protein yields were diminished, yeast cell pellets were examined for nonsecreted vpB. Significant amounts of vpB were not present in the crude lysate derived from lysed yeast cell pellets. Also the possibility of vpB adhering to the Centriprep membrane surface during the concentration process due to its adhesive property was investigated. However, no significant amount of vpB was recovered from Centriprep membranes which were first pre-treated with BSA (10 mg/ml), to minimize non-specific binding, and then washed with 0.1% and 1% SDS following concentration. It was also investigated whether residual ammonium sulfate remaining in the protein solution following dialysis might interfere with the BCA assay and

quantification of protein. Using a conductivity meter, the salt concentrations of each of the three ammonium sulfate fractions containing vpB protein were measured. The 40-60% and the 20-40% ammonium sulfate fractions each gave readings which corresponded to the 0.35 M and the 0.1 M standards, respectively. A group of ammonium sulfate standards, ranging in concentration from 0.01 M to 5 M, also were analyzed using the BCA assay and no interference by ammonium sulfate was observed.

3.1.1. Generation of Multi-Copy Inserts

Expression, isolation and purification of vpB using the *Pichia* system is a time-consuming and laborious process. To maximize the expected yield of foreign protein, our efforts are now focused on the use of a recently available vector, pPIC9K, which allows for the isolation of multicopy inserts by an *in vivo* method. This *in vivo* method utilizes resistance to G418 to screen for possible multi-copy inserts. Multiple plasmid integration events occur spontaneously in *Pichia* at a fairly low frequency (1-10% of all transformants). However, due to the presence of the bacterial kanamycin gene (*kan* from Tn903) in pPIC9K, which confers resistance to the antibiotic G418 in *Pichia*, there is now a tool available with which to identify these multiple insertion events. The vector is designed so that there is a genetic linkage between the kanamycin gene and the "expression cassette", and except for the presence of the kanamycin resistance gene, is otherwise identical to pPIC9 and functional in the same *Pichia* strain previously used. (Figure 2 illustrates multiple insertion of the expression cassette linked to the *kan* gene.) The degree of G418 resistance roughly corresponds to the number of kanamycin genes integrated. A single copy of pPIC9K integrated into the *Pichia* genome confers resistance to G418 at a level of approximately 0.25 mg/ml of G418. Increasing the copies of pPIC9K integrated increases the G418 resistance levels from 0.5 mg/ml (1-2 copies) to as high as 4 mg/ml (7-12 copies) G418. It is inferred that clones resistant to higher levels of G418 also contain multiple copies of the foreign gene (in our case, the gene encoding vpB).

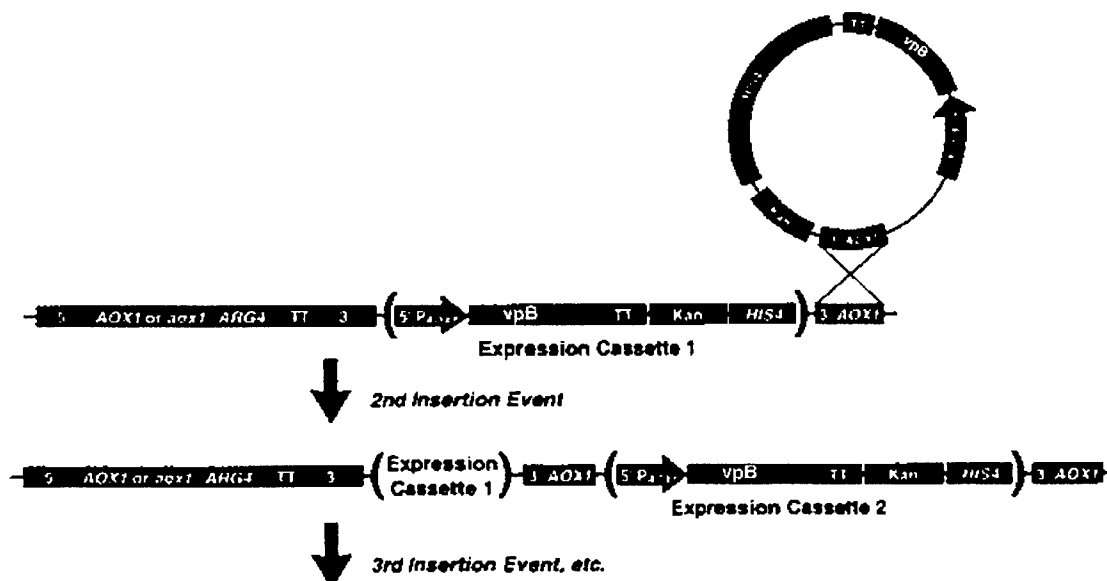


Figure 2. Graphic of multiple insertion of pPIC9K into *Pichia*.

During the past month, we have cloned the *F. hepatica* vpB gene into pPIC9K. vpB genomic DNA, isolated from a lambda dash genomic expression library, was PCR isolated and purified, and ligated into EcoR1 digested pPIC9K vector DNA. Recovered plasmid DNA was restriction digested and sequenced in order to determine correct orientation and reading frame before proceeding with transformation of the yeast. There are several options for transformation into *Pichia*. We have selected to use electroporation, in which electrocompetent *Pichia* cells are electrically pulsed in the presence of linearized, recombinant pPIC9K-vpB DNA. There are two methods used to screen transformants for G418 resistance. Both strategies involve growing transformed clones on varying concentrations of G418-containing plates and scoring for G418 resistance. Again, it is assumed that multiple copies of the foreign gene correspond to an increased level of resistance to G418 and will result in an increased amount of expressed protein. Once G418 resistant colonies are identified, they can be characterized for their methanol utilization phenotype (mut^t vs. mut^s) as well as for the copy number of the foreign gene. Our objective is to isolate a His⁺ *Pichia* transformant containing multiple copies of vpB enabling over-expression and secretion of vpB.

3.2. Optimization of Chitosan/Tripolyphosphate/BSA Nanosphere Preparation by Complex Coacervation

145 ml of 1.25 mg/ml chitosan was mixed with 55 ml water containing 0.3 mg/ml tripolyphosphate (TPP) and 1 mg/ml BSA. The resulting solution was clear with a pH of 4.1 with a final BSA concentration of 0.275 mg/ml. The solution was titrated with 5M NaOH up to pH 10.7 taking eight 2 ml aliquots at various pHs along the way. To half of the aliquot was determined the optical density at 570 nm to quantify the relative amount of light scattering particulate matter in solution and to the other half was determined the protein concentration in the supernatant (BCA from Pierce). The results of this experiment are plotted in Figure 3.

As the pH of the solution increases by the addition of base, the optical density of the solution increases and reaches a maximum at pH 6.9 and then begins to decline as the pH of the solution becomes more basic, while the concentration of soluble BSA (BSA in the supernatant of each aliquot) reaches a minimum (zero) at pH 6.9 and increases above neutral pH. Notice also that the maximum in optical density coincides with the minimum of soluble BSA. Another observation made during this experiment was physical characteristics of the particles in solution during the titration. Initially the solution was clear. As the pH increased the solution became cloudy but the particles formed were too small to be seen. Once the pH exceeded 7, the particles in solution appeared larger and quite irregular. These observations combined with the data shown in Figure 3 can be explained as follows:

In the mildly acidic range shown in Figure 3 BSA (pI = 5.4) has an overall neutral charge, chitosan is positively charged, and TPP is negatively charged. As the pH is increased through the neutral range BSA becomes more negatively charged and through non-specific electrostatic interactions associates with the positively charged chitosan. Although chitosan is positively charged it does begin to lose the amino proton of the repeating saccharide as the pH increases making the polymer less soluble. As a result, chitosan begins to come out of solution and it takes BSA and presumably some TPP with it. This happens slowly enough to generate nanospheres. However as the pH continues to rise, chitosan loses more and more protons until the microspheres are torn apart by the repulsive forces of the negatively charged BSA and TPP. This

results in BSA going back into solution and the appearance of irregular particles which are presumably ruptured nanospheres.

The previously described method for the preparation of chitosan/tripolyphosphate/BSA nanospheres¹² mixed the chitosan and tripolyphosphate (TPP) solutions at predetermined pHs. BSA was present in either the chitosan or TPP solution. Percent loading of BSA into the nanospheres was determined by subtracting the amount of BSA that remained in solution after nanosphere formation from the initial amount of BSA in solution. Loading efficiencies of 26%, 27% and 39% were obtained at pHs 3.0, 4.0 and 5.0 respectively¹². Figure 3 shows that by titrating to pH 6.9, 100% BSA can be loaded into chitosan/TPP nanospheres. Nanospheres have been prepared using this technique and were collected and lyophilized to dryness. At the time of this report SEMs of this preparation were not available.

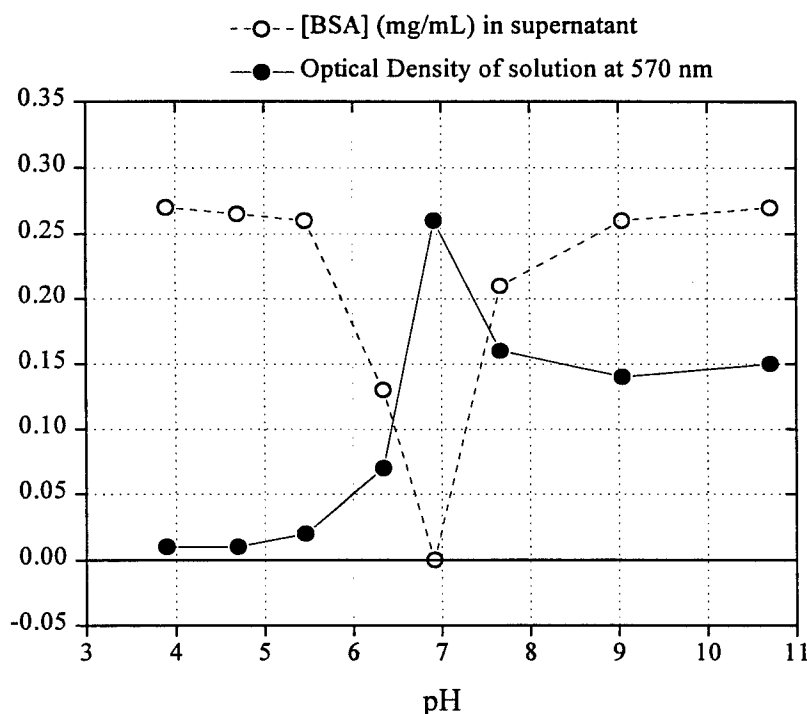


Figure 3. Formation of Chitosan/TPP/BSA Microspheres by Complex Coacervation

3.3. Microsphere Size and Surface Morphology Characterization

BSA-based microsphere preparations were made to determine how microsphere size was affected by impeller stirring speed and the type of emulsifying oil. BSA microspheres were prepared in hexadecane, olive oil, and castor oil at a variety of stirrer speeds ranging from 300 to 5000 rpm. The results are plotted in Figure 4. The lowest stirrer speed tested for each of the three oils was the minimum speed necessary to form an emulsion. The average microsphere diameter was qualitatively determined by measuring the diameter of ~20 microspheres under magnification using a bench-top light microscope. The conclusion from Figure 4 is that BSA-based microspheres ranging in size from 200 (± 60) μm to less than 10 (± 3) μm can be prepared

using the current microsphere preparation method. That the olive and castor oil curves appear to approach an asymptote of 10 μm is most likely artificial. Microspheres smaller than 3 μm are difficult to detect using our bench-top light microscope, therefore the asymptote is a reflection of the limitations of the optical sizing method and not of the microsphere preparation method. Subsequent scanning electron micrographs of BSA microspheres prepared in castor oil at 5,000 rpm revealed microsphere diameters ranging from 0.6 to 6 μm . Attempts to make submicron microspheres were not initially pursued since many literature references state that the optimum microsphere size for uptake by Peyer's patches is $\leq 10 \mu\text{m}$. Therefore subsequent microspheres were prepared in olive oil at 1,200 rpm.

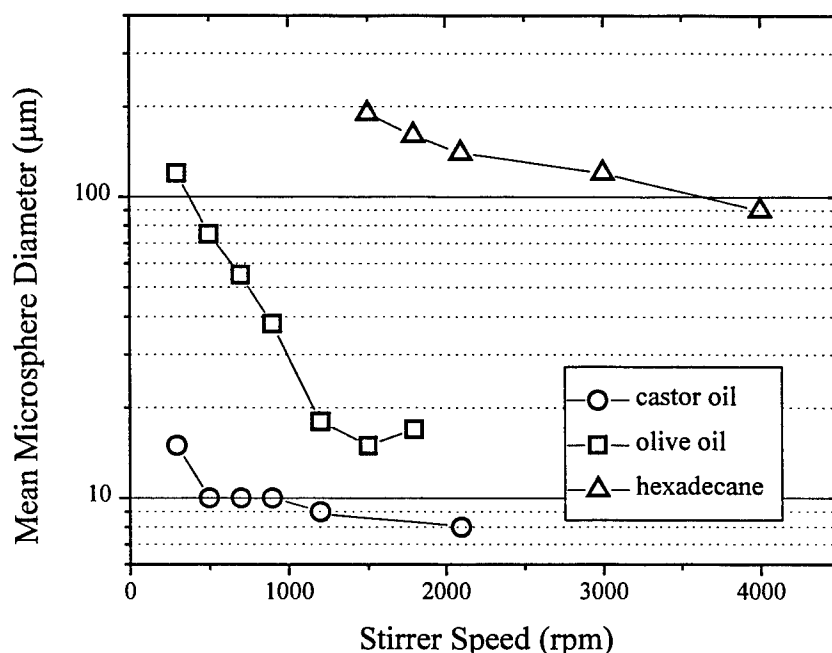
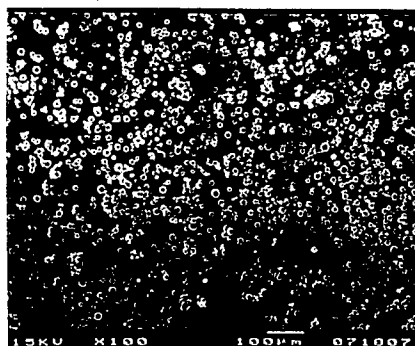


Figure 4. Microsphere size dependence on stirrer speed and emulsifying oil.

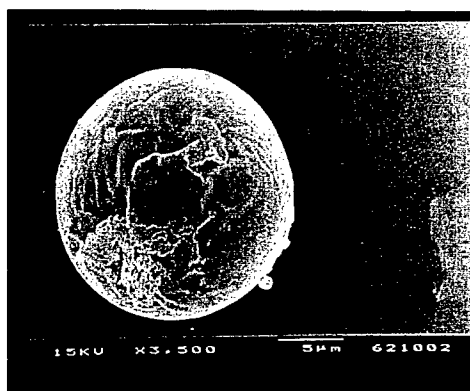
It was also observed that microsphere size was dependent on the temperature of the emulsion. Microspheres prepared in olive oil at 1,200 rpm were considerably larger when prepared at 58°C (~150 μm) than at room temperature (~10 μm). That temperature can affect microcapsule size is not surprising because higher temperatures can adversely affect protein stability and strengthen intermolecular forces. However, olive oil viscosity is inversely proportional to temperature (~ 24 centipoise at 58°C and ~ 84 centipoise at 23°C), therefore a more likely explanation is the lower viscosity oil (58°C) results in a lower surface tension at the water / oil interface, producing larger microspheres.

Figure 5 shows some typical microsphere preparations. It is apparent from Figure 5 that higher glutaraldehyde concentration yields microspheres with a smoother surface texture and presumably smaller pores, Figure 5. B and C, respectively. This presumption is corroborated by the *in vitro* release data (described below) which shows that microspheres crosslinked with a higher concentration of glutaraldehyde have slower release kinetics¹³.

(A)



(B)



(C)

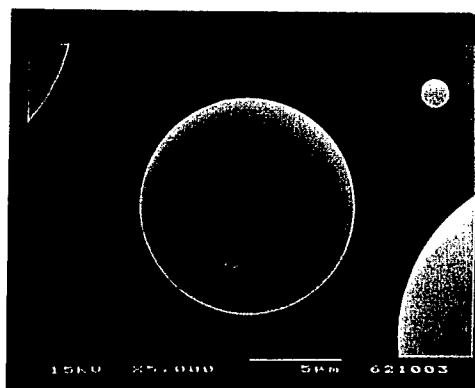


Figure 5. SEMs of three different BSA microsphere preparations. (A) 100 X magnification of microspheres prepared in castor oil at 600 rpm and crosslinked with 1 % glutaraldehyde. (B) 3,500X magnification of a 19 μm microsphere prepared in olive oil at 1200 rpm crosslinked with 1 % glutaraldehyde. (C) 5,000X magnification of a single 11 μm microsphere prepared in olive oil at 1200 rpm crosslinked with 25 % glutaraldehyde.

3.4. *In Vitro* Release Kinetics

In vitro release kinetics were determined for all four formulations prepared for *in vivo* Exp. I (described below in Table 1). The results of the *in vitro* release assay are shown in Figure 6. It is evident from the figure that Formulations 3 and 4 have a greater cumulative release over seven days than Formulations 1 and 2. That the release does not continue to increase at a uniform rate towards 100% but appears to approach an asymptote considerably less than 100% is anticipated. The release rate profile from crosslinked BSA microspheres is expected to be biphasic. The initial "burst" phase results from the release of BSA monomers and low molecular weight polymers from the surface and pores of the microsphere. The second "slow" phase is considerably slower because it requires breaking of covalent bonds to completely solubilize and release the remaining contents of the microsphere.

The *in vitro* release rate model should be fairly accurate for approximating *in vivo* release during the burst phase but probably not during the slow phase. The slow phase dissolution of the microsphere requires hydrolysis of the BSA peptide backbone which is thermodynamically favorable but kinetically unfavorable *in vitro*. However proteinases exist *in vivo* which catalyze peptide bond hydrolysis. Therefore, *in vivo*, the release rate during the slow phase will be much faster than predicted by the *in vitro* model.

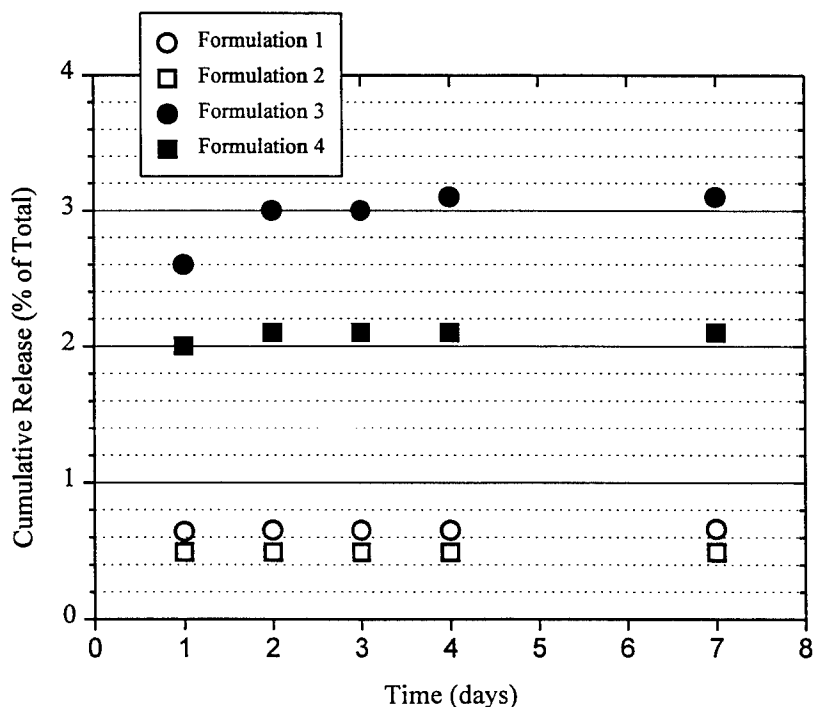


Figure 6. *In vitro* release from four microsphere formulations.

3.5. *In Vivo* Evaluation of Various Microsphere Formulations

A total of three animal experiments were performed to evaluate the *in vivo* immune response to Bot-Ac encapsulated in BSA/vpB microspheres. Animal Experiment I studied different formulations administered subcutaneously while Animal Experiments II and III compared identical formulations and doses administered either subcutaneously (Animal Experiment II) or orally (Animal Experiment III).

Animal experiments (IV and V) were also performed to refine techniques for determining microsphere uptake by M cells of the Peyer's patches. Below the results of all five animal experiments are described in detail.

3.5.1. Immune Response to Subcutaneously Administered Microspheres

3.5.1.1. Animal Experiment I

Animal Experiment I (Exp. I) was designed to determine the effects of three parameters on murine immune response to Bot-Ac: 1) microsphere size, 2) presence versus absence of vpB in the microsphere formulation, and 3) antigen dose. The four formulations in Table 1 plus a control (consisting of free Bot-Ac in buffer) were administered to eight to ten week old female Balb/c mice by subcutaneous injection between the shoulder blades distal to the head at three different dose levels.

Table 1. Microsphere Formulations Prepared for Exp. I

Formulation	% BSA / % vpB / % Bot-Ac	Prepared in Olive Oil at	Approximate Mean Diameter (μ m)
1	99.9 / 0 / 0.1	600 rpm	18
2	99.9 / 0 / 0.1	1500 rpm	8
3	89.9 / 10 / 0.1	600 rpm	22
4	89.9 / 10 / 0.1	1500 rpm	10

The fifteen animal groups in Exp. I and what they received are listed below:

- Group 1:** 6 mice received **Dose 1** (0.2 mg of Formulation 1 in 100 μ l buffer)
6 mice received **Dose 2** (0.6 mg of Formulation 1 in 100 μ l buffer)
6 mice received **Dose 3** (2.0 mg of Formulation 1 in 100 μ l buffer)
- Group 2:** 6 mice received **Dose 1** (0.2 mg of Formulation 2 in 100 μ l buffer)
6 mice received **Dose 2** (0.6 mg of Formulation 2 in 100 μ l buffer)
6 mice received **Dose 3** (2.0 mg of Formulation 2 in 100 μ l buffer)
- Group 3:** 6 mice received **Dose 1** (0.2 mg of Formulation 3 in 100 μ l buffer)
6 mice received **Dose 2** (0.6 mg of Formulation 3 in 100 μ l buffer)
6 mice received **Dose 3** (2.0 mg of Formulation 3 in 100 μ l buffer)
- Group 4:** 6 mice received **Dose 1** (0.2 mg of Formulation 4 in 100 μ l buffer)
6 mice received **Dose 2** (0.6 mg of Formulation 4 in 100 μ l buffer)

Group 5: 6 mice received **Dose 3** (2.0 mg of Formulation 4 in 100 μ l buffer)
6 mice received **Dose 1** (0.2 μ g of Bot-Ac in 100 μ l buffer)
6 mice received **Dose 2** (0.6 μ g of Bot-Ac in 100 μ l buffer)
6 mice received **Dose 3** (2.0 μ g of Bot-Ac in 100 μ l buffer)

The buffer in which all formulations were suspended and administered was 98% 10 mM PBS, pH 7.4 / 2% ethanol. Blood was collected from the tail of all mice (\sim 40 μ l) and blood from within a group and dose was pooled and a single serum IgG value was determined for each group at each dose by the standardized ELISA described above.

For this experiment, blood was collected and serum IgG levels were determined by the standardized ELISA protocol for all fifteen groups at 1, 2, 3, 4, 5, 7, 9, and 13 weeks and a 17 week data point was collected for all groups receiving dose 3. The groups that received Dose 1 and their serum IgG response is plotted in Figure 7, Dose 2 in Figure 8, and Dose 3 in Figure 9.

It is evident that at all three dose levels Group 5 (the control group in which free Bot-Ac was administered) displayed the greatest IgG response within the first few weeks. It is possible that the control is highest because all of the Bot-Ac administered to Group 5 is immediately available for immune processing. Microencapsulated formulations which contain the same amount of Bot-Ac as administered to Group 5 do not initially deliver the entire amount of antigen but slowly release the antigen over time.

The serum IgG levels from animals administered the lowest dose (0.2 mg microspheres containing a total of 0.2 μ g Bot-Ac, Figure 7) were low for the control group (Group 5) and all groups receiving microsphere formulations (Groups 1 through 4). Serum IgG levels from animals receiving the intermediate dose (0.6 mg microspheres containing a total of 0.6 μ g Bot-Ac, Figure 8) were higher for the control group and for Group 4 during the first five weeks however they also dropped to undetectable levels during weeks seven through thirteen. The best results were achieved with animals receiving the highest dose (2.0 mg microspheres containing a total of 2.0 μ g Bot-Ac, Figure 9). All groups receiving this dose produced detectable serum IgG during the first five weeks with the control and Group 4 once again showing the highest levels. More importantly is to notice that serum IgG drops to very low levels in the control group during weeks seven through eleven but serum IgG levels persist at relatively high levels in Group 4 up to seventeen weeks.

Closer examination of Figure 9 reveals that during the initial five week period, excluding the control group, comparative IgG levels were: Group 4 > Group 3 \approx Group 2 > Group 1. This implies that 10 μ m microspheres are better than 20 μ m microspheres and that microspheres containing vpB are better than those that do not. 20 μ m microspheres that do contain vpB elicit similar serum IgG levels as 10 μ m microspheres that do not contain vpB.

Figure 10 replots the Group 4 serum IgG response from all three doses. It is evident from Figure 10 that Dose 3 generates the highest serum IgG level followed by Dose 2 and then Dose 1. Closer inspection of Figures 7 through 9 will also show this for Groups 1 through 3. The observation that more antigen is better for subcutaneously injected microspheres certainly holds true in the 0.2 to 2.0 μ g antigen per animal range. Higher antigen doses will be administered in future *in vivo* experiments to find the maximum of the dose versus serum IgG response relationship.

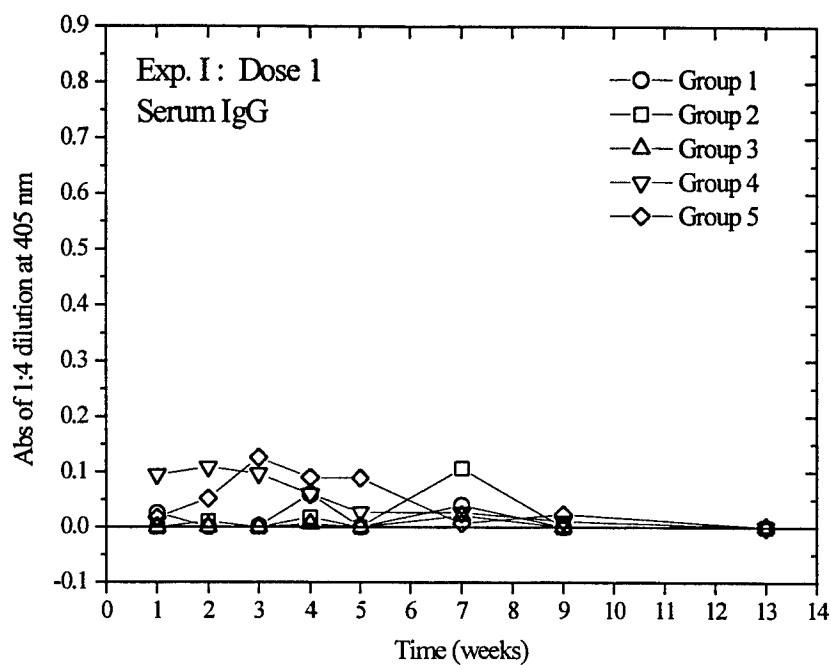


Figure 7. Exp. I: Serum IgG response to the five groups receiving Dose 1

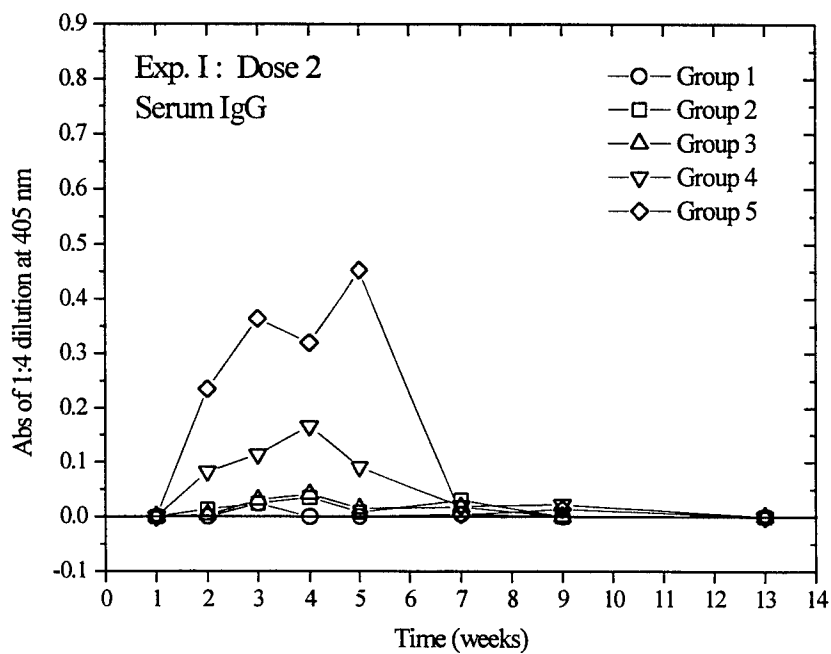


Figure 8. Exp. I: Serum IgG response to the five groups receiving Dose 2

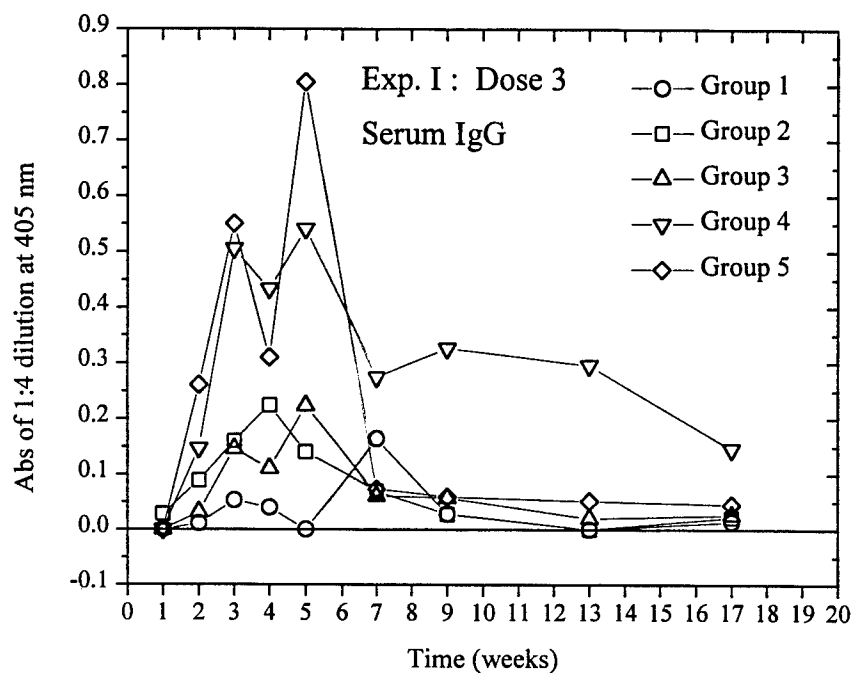


Figure 9. Exp. I: Serum IgG response to the five groups receiving Dose 3

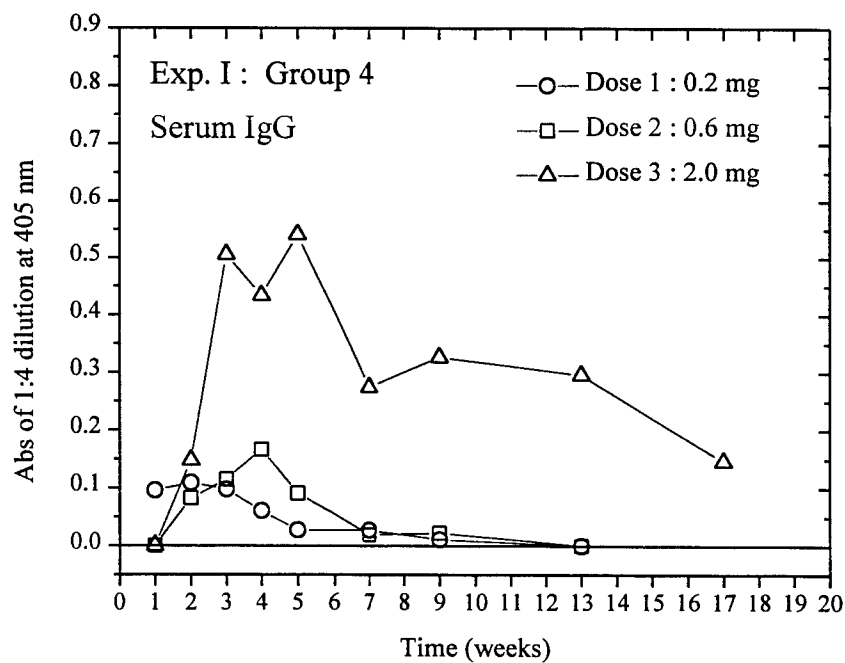


Figure 10. Exp. I: Dose dependent Serum IgG response of Group 4

3.5.1.2. Animal Experiment II

The results from Exp. I showed that 10 μ m microspheres generated higher serum IgG levels than 20 μ m microspheres and that a dose of 2.0 mg was better than either 0.6 or 0.2 mg. This information was used to design Exp. II to determine the influence of two additional parameters: 1) amount of glutaraldehyde crosslinking of microspheres, and 2) presence versus absence of vpB at the new glutaraldehyde crosslinking levels. In Exp. I, 1.5% glutaraldehyde was used. In Exp. II 1.5 % glutaraldehyde is repeated along with 1.0 and 0.5%. Lower glutaraldehyde amounts produce microspheres that release antigen faster in *in vitro* release experiments¹³.

The six formulations in Table 2 plus a control (consisting of free Bot-Ac in buffer) were administered to eight to ten week old female Balb/c mice by subcutaneous administration.

Table 2. Microsphere Formulations Prepared for *In Vivo* Exp. II

Formulation	% BSA / % vpB / % Bot-Ac					% Glutaraldehyde Used to Crosslink Microspheres
1	89.9	/	10	/	0.1	1.5
2	89.9	/	10	/	0.1	1.0
3	89.9	/	10	/	0.1	0.5
4	99.9	/	0	/	0.1	1.5
5	99.9	/	0	/	0.1	1.0
6	99.9	/	0	/	0.1	0.5

The seven animal groups and what they received are listed below:

- Group 1 : 6 mice received 2.0 mg of Formulation 1 **subcutaneously** in 250 μ l buffer
- Group 2 : 6 mice received 2.0 mg of Formulation 2 **subcutaneously** in 250 μ l buffer
- Group 3 : 6 mice received 2.0 mg of Formulation 3 **subcutaneously** in 250 μ l buffer
- Group 4 : 6 mice received 2.0 mg of Formulation 4 **subcutaneously** in 250 μ l buffer
- Group 5 : 6 mice received 2.0 mg of Formulation 5 **subcutaneously** in 250 μ l buffer
- Group 6 : 6 mice received 2.0 mg of Formulation 6 **subcutaneously** in 250 μ l buffer
- Group 7 : 6 mice received 2.0 μ g of free Bot-Ac **subcutaneously** in 250 μ l buffer

The buffer in which all formulations were suspended and administered was 93% 10 mM PBS, pH 7.4 / 5 % 20 mg/ml SDS / 2% ethanol. Blood was collected from the tail of all mice (~40 μ l) and saliva was collected from all mice following a 100 μ l interperitoneal injection of 1 mg/ml pilocarpine. Blood and saliva from within a group were pooled and a single serum IgG, serum IgA and saliva IgA value were determined for each group.

The results from Exp. II are shown in Figures 11 through 13. Figure 11 shows the serum IgG response to subcutaneously administered microspheres. Consistent with results from Exp. I, Exp. II shows that mice receiving microspheres that contain vpB (Groups 1 through 3) show a higher serum IgG level than mice receiving microspheres that do not (Groups 4 through 6). *In vitro* release experiments with 100% BSA microspheres crosslinked with 1.0 and 1.5% display a

considerable difference in release rate (20% and 4%, respectively after four days¹³) but at five weeks a significant difference is not observed *in vivo* between microspheres crosslinked with 0.5, 1.0, or 1.5 % glutaraldehyde. Since immune response is dependent on antigen release rate (i.e. dose, see results from Exp. I) from the microspheres, then incubating BSA microspheres in PBS appears to be a poor *in vitro* model for the *in vivo* environment. Unlike microspheres prepared from some synthetic polymers (i.e. poly(lactide-co-glycolide) and poly(ϵ -caprolactone)) which degrade by base catalysis and is therefor primarily pH dependent, BSA microspheres, like other protein, based microspheres degrade by enzymatic degradation. Therefor any attempt at a reasonable *in vitro* model for protein based microspheres would require a cocktail of physiologically relevant proteinases at physiologically appropriate concentrations in the PBS dissolution medium.

Serum and saliva IgA (Figures 12 and 13) were expected to be and are considerably lower than serum IgG (Figure 11). Nevertheless these levels were determined in order to make direct comparisons to Exp. III in which mice orally received the same formulations as mice subcutaneously received in Exp. II.

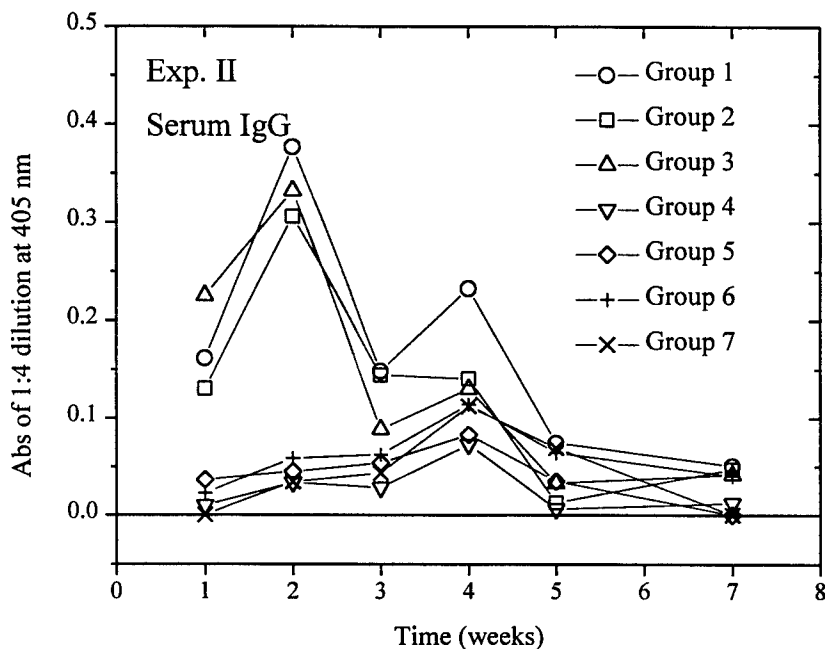


Figure 11. Exp. II: Serum IgG response to subcutaneously administered microspheres

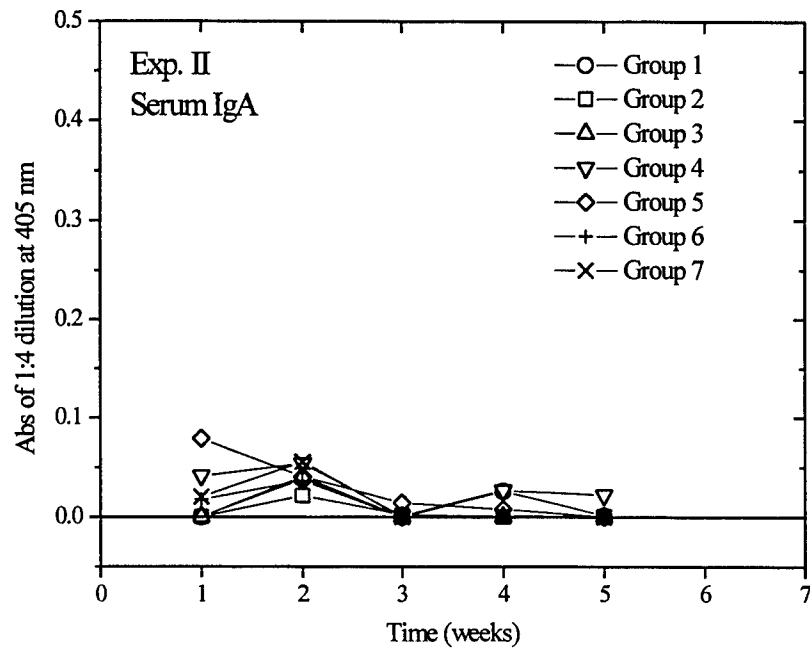


Figure 12. Exp. II: Serum IgA response to subcutaneously administered microspheres

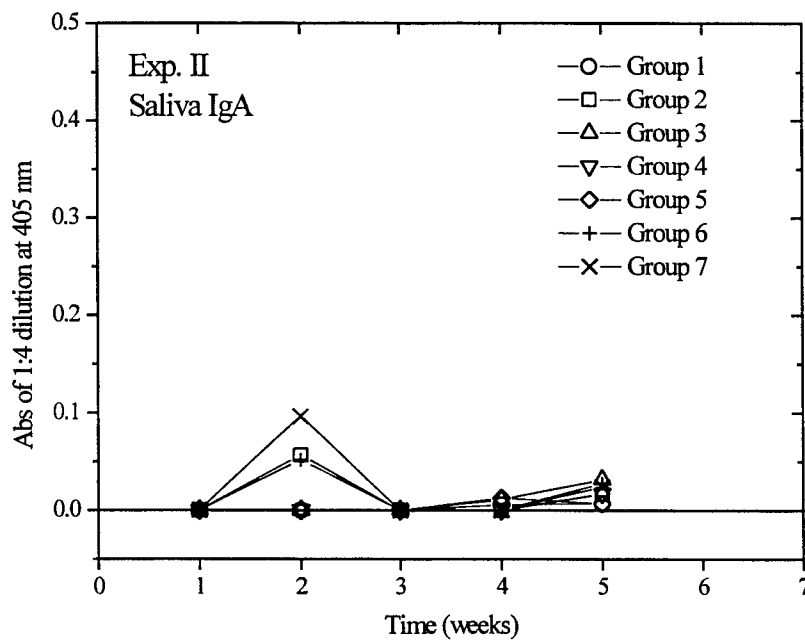


Figure 13. Exp. II: Saliva IgA response to subcutaneously administered microspheres

3.5.2. Immune Response to Orally Administered Microspheres

3.5.2.1. Animal Experiment III

The same formulations and doses that were administered to animals subcutaneously in Exp. II were administered orally in Exp. III. This was done to provide a direct comparison, independent of formulation and dose, between the two administration routes. The results from Exp. III are shown in Figures 14 through 16. The best serum IgG response was obtained from Groups 2 and 3 which were two of the highest obtained from subcutaneous administration in Exp. II. Both of these formulations contained 10% vpB at different glutaraldehyde crosslinking concentrations (see Table 2). In general, the overall immune response to orally administered microspheres was considerably less than those administered subcutaneously.

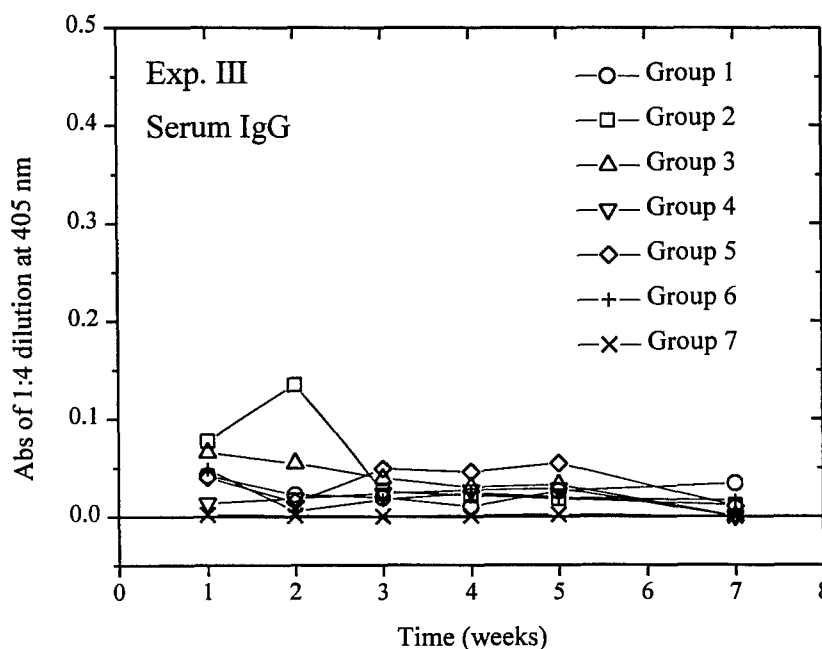


Figure 14. Exp. III: Serum IgG response to orally administered microspheres

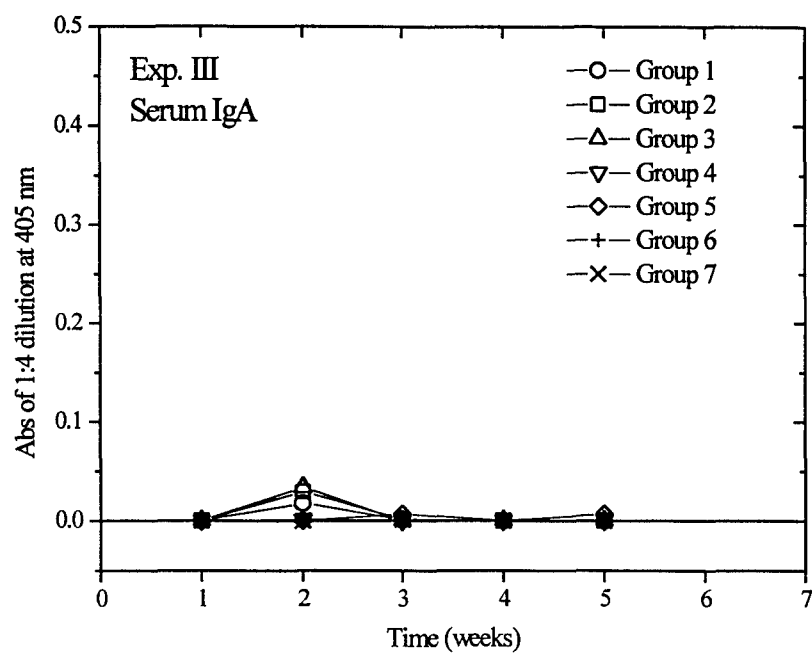


Figure 15. Exp. III: Serum IgA response to orally administered microspheres

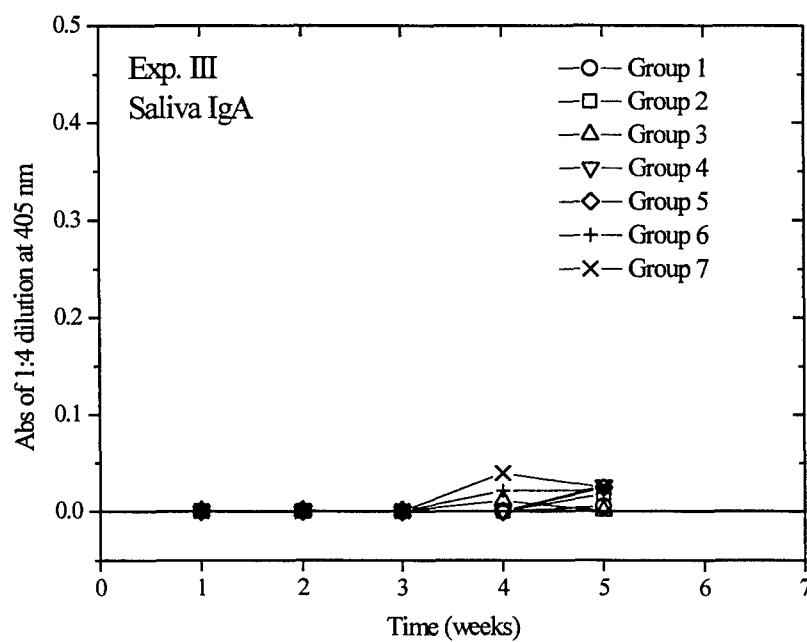


Figure 16. Exp. III: Saliva IgA response to orally administered microspheres

Once it was determined that oral administration of microspheres generated a low secretory (salivary) immune response it was questioned whether the absence of proteinase inhibitors and metal chelators could have contributed to proteolytic degradation of the immunoglobulins during storage. Some examples of saliva storage in the literature use inhibitors while others do not. To determine if immunoglobulins were destroyed during storage a control experiment was performed. A known amount of anti-Bot-A was used to spike fresh murine saliva and PBS. After a one week storage at -20°C the titer of each was determined and compared. No significant difference was observed between the two samples suggesting that degradation of immunoglobulins in the saliva during storage does not occur.

3.5.3. Peyer's Patch Uptake of Orally Administered Microspheres

3.5.3.1. Animal Experiment IV

The immune response elicited by oral administration of Bot-Ac loaded BSA/vpB microspheres was considerably lower than anticipated. It was then necessary to determine whether this was because of poor Peyer's patch uptake or some other microsphere formulation based problem (i.e. release kinetics, etc.). Therefore it was necessary to compare BSA/vpB microsphere uptake to another well adsorbed material such as polystyrene. However a preliminary experiment was performed to work out technical problems for subsequent Peyer's patch uptake experiments. Sixteen 8 to 10 week old female Balb/c mice were fasted for 16 hours. Each mouse was then orally administered 2 mg of 8 μ m polystyrene microspheres in 250 μ l of water (estimated at 7.5×10^6 microspheres/dose). This dose was selected to be the same as that delivered in Exp. III although polystyrene and vpB/BSA microspheres were not expected to have the same or even similar Peyer's patch uptake efficiencies. Two mice each were sacrificed at 15, 30, 60, 90, & 120 minutes and one mouse each at 3, 4, 5, 24, 48, 72 hours. From each animal was collected the stomach, small intestines (up to but excluding caecum), spleen, liver, lungs, and mesenteric lymph nodes. The tissues were fixed in buffered formalin and stained. However, when viewed under a standard bench top light microscope, no microspheres were observed to penetrate Peyer's patches or other surrounding tissues.

3.5.3.2. Animal Experiment V

A second Peyer's patch experiment was started. Twenty Balb/c mice were fasted for sixteen hours and divided into four groups of five each. To each mouse in Group 1 was orally administered 40 mg of 3 μ m diameter polystyrene microspheres in 200 μ l water (estimated at 2.9×10^9 microspheres/dose). To each mouse in Group 2 was administered 20 mg of 6 μ m diameter polystyrene microspheres dyed red in 200 μ l water (estimated at 1.9×10^8 microspheres/dose). To each mouse in Group 3 was administered 20 mg of 3 μ m diameter polystyrene microspheres dyed yellow in 200 μ l water (estimated at 1.4×10^9 microspheres/dose). To each mouse in Group 4 was administered 4 mg of 6 μ m diameter BSA microspheres in 200 μ l water (estimated at 1.9×10^8 microspheres/dose). One animal from each group was sacrificed at 2, 4, 24, 48 and 72 hours. The same organs mentioned in Exp IV were collected, fixed and stained. At the time of the writing of this report no data was available from this experiment.

4. Discussion

The first step involved the preparation of microencapsulated Bot-Ac. Bot-Ac was encapsulated in BSA microspheres by the water-in-oil polymerization technique. Microspheres ranging from 3 to 200 μm were prepared using this technique and it was demonstrated that microsphere size was *directly* proportional to the temperature of the emulsion and *inversely* proportional to both stirrer speed and the viscosity of the emulsifying oil (suspension medium). The latter two observations are consistent with the following proportionality, which is a qualitative guide for controlling the size of microspheres produced by the water-in-oil polymerization technique¹⁴.

$$\bar{d} \propto K \frac{D_v R v_d \gamma}{D_s N v_m C_s}$$

where \bar{d} is the average droplet (particle) size; K symbolizes parameters such as vessel design and type of stirrer; D_v is the diameter of the vessel; D_s is the diameter of the stirrer; R is the volume ratio of the droplet phase to suspension medium; N is the stirring speed; v_d is the viscosity of the droplet phase; v_m is the viscosity of the suspension medium; γ is the surface tension between the two immiscible phases; and C_s is the stabilizer concentration. Although the temperature of the emulsion does not appear in the above proportionality, it is expected to have a strong influence on particle size because parameters such as v_d , v_m , and γ are temperature dependent.

It was also demonstrated that the *in vitro* release rate of total protein from microspheres was more influenced by the composition of the microspheres than the size of the microspheres for the size range studied. Formulations 3 and 4 from Exp. I both contained vpB and released protein much faster than those that contained no vpB. The primary target of glutaraldehyde crosslinking is primary amino groups located in the protein which are specifically lysyl amino acid side chains. vpB has a slightly higher lysine content than BSA (12%¹⁵ versus 10% respectively). A higher lysine content could result in a higher percentage of the glutaraldehyde being consumed in an unproductive manner by increasing intramolecular crosslinks. Consequently, microspheres that contain vpB could have less intermolecular crosslinks and therefore faster release kinetics.

Exp. I clearly demonstrated that immune response is strongly dose dependent with little to no response at the lowest dose (0.2 mg) and successively better responses at higher doses (0.6 and 2.0 mg). In addition the formulations that produced the greatest immune response were those that had the fastest release kinetics in the *in vitro* release study. Consequently, formulations for Exp. II were prepared at the 2.0 mg dose level and to have even greater release kinetics to elicit a greater immune response. The amount of glutaraldehyde crosslinking was varied downward from 1.5 to 0.5% which considerably increases the *in vitro* release rate¹³. However the results from Exp. II demonstrate that varying glutaraldehyde crosslinking from 1.5 to 0.5% does not change the immune response. This suggests that either the *in vitro* release model is poor at estimating the *in vivo* release kinetics or that release rates above those achieved with the 1.5% glutaraldehyde crosslinking do not enhance immune response further.

Exp. III which orally administered the same microsphere formulations subcutaneously administered in Exp. II elicited a smaller immune response. Unlike subcutaneous administration

(Exps. I & II) which specifically delivers the entire dose directly to the target area, the target tissue of oral administration is the lymphoid aggregates, termed Peyer's patches, imbedded along the antimesenteric aspect of the small intestine. Oral administration of 2.0 mg of microspheres by gavage technique will certainly result in a lower *effective* dose for two main reasons:

- 1) only microspheres with a diameter of $\leq 10 \mu\text{m}$ are absorbed by Peyer's patches. All orally administered microspheres formulations in this experiment had an average diameter of $10 \mu\text{m}$; therefore only 50% (assuming a symmetrical size distribution) of the administered microspheres were of the appropriate size for Peyer's patch uptake, and
- 2) the efficiency of particle uptake by Peyer's patches is considerably less than 100%.

Damge and coworkers determined the uptake efficiency of rhodamine B labeled poly(lactide-co-glycolide) microspheres of 1-5 μm and 5-10 μm were 12.7% and 0.11% respectively¹⁶. Many other examples also exist where 10^7 to 10^9 microspheres are orally administered but only hundreds or at most thousands are ever identified in the Peyer's patches, lymph nodes or other organs^{17,18,19,20}.

In addition, the greatest immune response observed in the subcutaneous administration experiments (Exps. II & III) was serum (or circulating) IgG. It's been postulated that only orally administered microspheres $\leq 5 \mu\text{m}$ can stimulate a circulating immune response because of their propensity to disseminate to systemic lymphoid tissues within antigen presenting accessory cells. Therefore the effective oral microsphere dose with a potential to elicit circulating antibodies is proportional to the fraction of microspheres $\leq 5 \mu\text{m}$ times the uptake efficiency. This will certainly produce an effective dose much less than the administered 2.0 mg.

Subsequent oral animal experiments will use microsphere preparations that have an average diameter of $\sim 5 \mu\text{m}$ which will increase the effective dose and should elicit a greater immune response. Also, a separate dose versus response profile will be required for oral administration.

It was anticipated that a 2 mg dose of 8 μm polystyrene microspheres orally administered in water would result in detectable microspheres in the Peyer's patches. That no microspheres were observed in the Peyer's patches from Exp. IV was certainly unanticipated. As a result a second Peyer's patch experiment was started (Exp. V) that increased the dose by 10 to 20 times and decreased the particle size from 8 to 3 μm . In addition, both red and yellow dyed polystyrene microspheres were administered to enhance visualization. All of these factors should considerably enhance polystyrene microsphere uptake and detection. The results from Exp. V are still unavailable.

Entrapment of BSA into chitosan/tripolyphosphate microspheres was performed as a preliminary test to evaluate the complex coacervation system for chitosan-based microsphere preparation. This system has an advantage over the conventional water in oil emulsion polymerization technique in that the water in oil polymerization technique exposes microsphere proteins (including the proteinaceous antigen) to the water/oil interface. Proteins at that interface will decrease their chemical potential by unfolding and exposing their hydrophobic core to the oil. This results in a very hydrophobic microsphere surface which makes the microspheres difficult to suspend and administer in a limited volume usually required by gavage techniques. (That the protein of interest denatures would be detrimental for most biochemical delivery

systems but is probably not so for vaccine delivery since the antigen ultimately gets processed into antigenic peptides and then presented in a complex with class II MHC.). It has been reported that Peyer's patch uptake is directly proportional to microsphere hydrophobicity suggesting that chitosan/TPP microspheres are less likely to be absorbed as well as BSA microspheres prepared by the water-in-oil polymerization technique. However, the ability to deliver a much higher dose may offset the loss due to surface hydrophobicity and generate a greater effective dose.

5. Conclusions

The conclusions of the first year work on this project are itemized below:

1. The bioadhesive protein, vpB, can be produced at 175-350 mg/L in a *Pichia pastoris* expression system;
2. BSA/vpB microspheres with diameters between 0.5 and 200 μm can be prepared using the oil in water polymerization technique;
3. A direct correlation between the *in vitro* microsphere release data and *in vivo* immune response does not exist suggesting that the *in vitro* release model is a poor representation of the *in vivo* environment;
4. 10 μm microspheres generate a higher serum IgG level than 20 μm microspheres when administered subcutaneously;
5. Microspheres that contain 10% vpB generate a higher serum IgG level than microspheres that do not contain vpB when administered either orally or subcutaneously;
6. Higher doses within the range of 0.2 to 2.0 μg Bot-Ac generate higher serum IgG levels when administered subcutaneously;
7. 2.0 mg of 10 μm microspheres containing 89.9% BSA / 10.0% vpB / 0.1% Bot-Ac generated a detectable serum IgG level at 17 weeks post-immunization.

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
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